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New and emerging roles of small RNAs in neurodegeneration, muscle, cardiovascular and inflammatory diseases

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Summary

Small noncoding RNAs (snRNAs) were discovered more than two decades ago, yet it was not until relatively recently that their important role in genome regulation was recognised. With such a substantial role in genome regulation, it is not surprising that snRNAs are crucial contributors to an ever-increasing number of diseases, as evidenced by the long list of published studies. Currently, microRNAs (miRNAs) represent the most intensively studied snRNAs. Dysregulation of miRNAs has been confirmed in numerous diseases, and changes in their levels could play an essential role in disease onset and progression and could be used for prognosis and potential therapy. Indeed, disease-altered miRNAs may either signify a direct trigger or a consequence of the disease. Therefore, miRNAs represent unique targets for disease intervention through their down- or up-regulation. Importantly, miRNAs may facilitate disease monitoring by detection of disease-altered miRNAs in easily accessible bodily fluids, such as blood or cerebrospinal fluid. Therefore, study of these events is of utmost importance for understanding the molecular mechanisms that drive disease, as well as for diagnosis and therapy. Here we attempted to synthesise a large number of studies to highlight the crucial role of miRNAs in the pathogenesis of neurodegenerative, muscle, cardiovascular and inflammatory diseases.

Key words: *small noncoding RNAs; microRNAs; RNA misregulation; genome regulation; neurodegeneration; muscle disease; cardiovascular disease; inflammatory diseases; biomarkers*

Introduction

While the incredible race to sequence the human genome was in progress, it was predicted and anticipated that humans would have more protein-coding genes than, for instance, the mouse [1, 2]. The results were therefore quite

surprising, since only about 1%–2% of the human DNA is ‘coding’ [3]. The rest was given names such as ‘noncoding’ or ‘junk’ DNA and it was not until relatively recently that the majority of this ‘junk DNA’ was acknowledged to be transcribed [4–6] and to have a function [7]: indeed, a function that has changed our view of gene expression, genome regulation and even evolution, development and the ever-fascinating complexity of the brain and its rapid expansion in primates [8, 9].

The first findings came about 20 years ago, when miRNA in *Caenorhabditis elegans* was found to regulate gene expression or, more precisely, messenger RNA (mRNA) translation [10]. The miRNAs were the first genome regulatory small noncoding RNAs to be discovered and since then, various noncoding RNA species with multiple functions have been identified [7].

Small noncoding RNAs (snRNAs) are present during evolution, and the more complex the organism, the more species of snRNAs develop (such as small interfering RNAs [siRNAs], piwi-interacting RNA [piRNAs] and miRNAs). It is believed that they originally served as genome protectors against endogenous and exogenous threats and that they acquired their genome regulatory functions later in evolution [8]. Long noncoding RNAs (lncRNAs) arrived on the scene of gene regulation relatively recently but their function appears to be connected to increased evolutionary speed [8, 9]. The lncRNAs retain many of the protein-coding gene characteristics but are expressed with tighter tissue specificity and are mainly nuclear localised [3]. These differences indicate their function, as a new regulatory layer responsible for fine-tuning the snRNAs [8] as demonstrated by the numerous interactions between lncRNAs and miRNAs [11–13].

Box 1**Main enzymes of the miRNA pathway**

Drosha – Enzyme that cleaves long primary pri-miRNA transcripts generating approx. 70bp pre-miRNAs

Dicer – Enzyme that cleaves double-stranded RNA and pre-miRNA into short double-stranded siRNAs and miRNAs

RISC – RNA-induced silencing complex, a multiprotein complex that incorporates one strand of either siRNA or miRNA which then serves as a template for perfect mRNA binding in case of siRNAs or for binding to imperfect complementary sites within the 3'UTRs of miRNA target mRNAs

Argonaute – Member of RISC, an enzyme that cleaves the target mRNA leading to RNA interference by mRNA degradation

Among the noncoding RNAs (ncRNAs), miRNAs are the most extensively studied and over 2 000 miRNAs have so far been identified in humans, each having several hundreds of potential target mRNAs [14]. In addition, thousands of single nucleotide polymorphisms (SNPs) were discovered within miRNA sites [15] and length and sequence heterogeneity (IsomiRs) for the vast majority of miRNAs has also been found [16]. Such polymorphisms might therefore contribute to phenotypic differences or even human diseases [15, 17, 18]. With such an extensive involvement in gene regulation, it is of no surprise that malfunctions of noncoding RNAs have been found to be directly responsible for or contribute to the pathogenesis of numerous disorders [8, 9, 17, 19]. These disease-inducing changes could be used for diagnosis, as alterations in miRNAs could be detected in cerebrospinal fluid or even blood plasma [17]. Moreover, they could be used as therapeutic targets [20, 21] with high potential, as a single miRNA could have hundreds of targets [14]. Furthermore, a major technological advancement originating from the noncoding RNA field is the broadly used downregulation of gene(s) of interest via small interfering RNA mechanism. In conclusion, noncoding RNA research is gaining momentum and we anticipate that while uncovering the ncRNA interactions with DNA, RNA and proteins, we will witness numerous, as yet unexplained, mechanisms becoming the solutions for many diseases.

Here, we review the latest discoveries on the role of miRNAs in human disease, focusing on neurodegenerative diseases, skeletal muscle and cardiovascular diseases, and the pathogenesis of inflammation.

Small noncoding RNAs in neurodegenerative diseases

Since the hallmark of most neurodegenerative diseases is protein aggregation [22], research has mainly been focused on the study of these misfolded and aggregated protein species. Hence, the term 'proteinopathies' has been established. Recent discoveries, however, show that major neurodegenerative diseases also involve miRNA dysfunction (table 1). Their importance for brain development and

function was first noted in Dicer knockout experiments, resulting in absence of mature miRNAs and siRNAs, which led to widespread neuronal alterations during development. Dicer knockout is embryonically lethal in mice [23] and its conditional inactivation in defined neuronal populations or brain regions results in an abnormal phenotype and neuronal degeneration [24, 25]. Moreover, cell-specific Dicer ablation in oligodendrocytes [25] or astrocytes [26] severely affected miRNA biogenesis in these cells leading to their malfunction, which ultimately resulted in noncell autonomous neuronal dysfunction and degeneration, emphasising the role of both miRNAs and glial cells in neurodegeneration. Further work revealed the involvement of miRNA and siRNA in neuronal stem cell differentiation, cell-to-cell interaction, neural development, axonal growth and guidance, as well as synaptic transport [19, 26, 27]. Moreover, piwi-interacting RNAs (piRNAs) and small-nucleolar RNAs (snoRNAs) were shown to contribute in the regulation of complex neuronal functions, such as learning and memory formation [15, 28, 29].

Although the links between the dysregulated miRNAs in neurodegenerative diseases and their target mRNAs or their regulatory lncRNAs are still poorly understood, new exciting discoveries highlight the role of miRNAs in the pathogenesis of neurodegenerative diseases (fig. 1). Importantly, miRNAs reflecting the disease stage have been identified in plasma and whole blood samples, indicating their potential use as biomarkers that are desperately needed. In addition, given the large number and high stability of circulating miRNAs, it is possible that through use of unbiased high-throughput screenings, miRNAs could also be used to differentiate neurodegenerative diseases from other conditions, e.g., inflammatory neurological diseases. In the following sections, we describe the role of the most important miRNAs involved in Alzheimer's disease, Parkinson's disease, prion disease, amyotrophic lateral sclerosis, frontotemporal dementia and Huntington's disease.

Box 2**Staging of neurodegenerative diseases**

Braak stages – AD staging that is based on the distribution of tau neurofibrillary tangles (Braak I-VI). Braak staging was also developed for PD where it is based on distribution of Lewy bodies

HD grades – HD or Vonsattel grades are used for HD staging and are based on the severity and pattern of striatal degeneration (HD 0-4)

Alzheimer's disease

Alzheimer's disease (AD) is an age-related progressive dementia and is the most common neurodegenerative disease. With a worldwide prevalence estimated to be 24 million [31], AD is one of the most common diseases in the industrial world [30]. AD is clinically characterised by a slow but progressive loss of memory and cognitive functions that leads to dementia [32] and ultimately to death [33]. Pathological hallmarks of the disease are the extracellular senile plaques composed of aggregated and misfolded

ded β -amyloid ($A\beta$) peptide and intracellular neurofibrillary tangles made of hyperphosphorylated tau protein, accompanied by microvascular damage and neuroinflammation [33]. Vast and broad loss of brain weight and volume is most likely caused by shrinkage and subsequent loss of neuronal processes, as well as specific loss of neurons within the entorhinal cortex and the hippocampus [34]. The majority of AD cases (>95%) do not show clear inheritance and they are called sporadic or late-onset AD. Nevertheless, several genetic factors were shown to contribute to the development of sporadic AD [33]. On the other hand, mutations in the amyloid precursor protein (APP), presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*) all lead to aggregation of the $A\beta$ peptide and cause the early-onset, autosomal dominant form of AD [30] with Mendelian inheritance.

miRNA-mediated regulation of the amyloid precursor protein

Numerous studies of miRNAs in AD have been performed both in AD models and human AD patient samples. It is clear that miRNAs are deregulated in AD [35] and that miRNA levels change according to disease stage [36, 37] or AD type [38]. Direct APP mRNA regulation and subsequent decrease of $A\beta$ accumulation has been shown for miR-101 *in vitro* [39, 40] and for miR-384 both *in vitro* and in cerebrospinal fluid (CSF) and serum of AD patients [38]. In addition, miR-384 also targets and regulates BACE1 (β -secretase 1; β -site APP cleaving enzyme 1), which in turn also indirectly represses the $A\beta$ load, as a negative correlation between miR-384 and $A\beta_{42}$ was observed in serum and CSF of AD patients [38]. Several other miRNAs (miR-20a, miR-17, miR-147, miR-655, miR-323-3p, miR-644,

Table 1: Reported miRNA alternations in neurodegenerative diseases.

Disease	Dysregulation in patient samples			
	Up in CNS	Down in CNS	Up in periphery	Down in periphery
Alzheimer's disease	miR-26b (temporal cortex)[56] miR-9, miR-128 (CA1 hippocampus)[58]	miR-124 (anterior temporal cortex)[42] miR-107 (superior and middle temporal cortex)[37] miR-29a, miR-29b-1 (high BACE1 brain)[50] miR-29a (cerebral cortex)[53] miR-132-3p (prefrontal cortex)[57] miR-146b (hippocampus, frontal gyrus)[36]	miR-9, miR-125b, miR-146a, miR-155 (CSF, neocortex ECF) [43] miR-34a, miR-181b, let-7f, miR-200a (blood mononuclear cells)[54] let-7d-5p, let-7g-5p, miR-15b-5p, miR-142-3p, miR-191-5p, miR-301a-3p and miR-545-3p (plasma)[59]	miR-384 (blood, CSF)[38]
Parkinson's disease		miR-133b (midbrain)[69] miR-205 (frontal cortex, striatum)[73]	miR16-2*, miR26a2* (peripheral blood early onset patients)[78] miR-331-5p (plasma)[79]	miR1, miR-22*, miR-29a (peripheral blood non-treated patients)[78] miR-126* (peripheral blood mononuclear cells)[237]
Amyotrophic lateral sclerosis	miR-624, miR-520e, miR-524-5p, miR-548a-5p, miR-606, miR-612, miR-647 (sporadic ventral lumbar spinal cord)[136] miR-b1123, miR-b2948, miR-b3265, miR-b5539, miR-sb1217*, miR-sb3998 (novel miRNA with MREs in NEFL mRNA 3'UTR, sporadic ventral lumbar spinal cord)[137] miR-24-2*, miR-142-3p, miR-142-5p, miR-146a, miR-146b, miR-155 (spinal cord)[163]	miR-9, miR-9-2 (human iPSC-derived neurons)[134] 239 different miRNAs (sporadic ventral lumbar spinal cord)[136] miR-b1336, miR-b2403, miR-b4652, miR-sb659* (novel miRNA with MREs in NEFL mRNA 3'UTR, sporadic ventral lumbar spinal cord)[137]	miR-23a, miR-29b, miR-206, miR-455 (skeletal muscle)[151] miR106b, miR-206 (serum)[152] miR-338-3p (sporadic peripheral blood leucocytes)[167] miR-143-5p, miR-574-5p (sporadic CSF)[168]	miR-451, miR-1275, miR-328-5P, miR-638, miR-149, miR-655 (sporadic peripheral blood leucocytes)[167] miR-132-5p, miR-132-3p, miR-143-3p (sporadic CSF)[168] miR-132-5p, miR-132-3p, miR-143-5p, miR-143-3p, let-7b (sporadic serum)[168] miR-574-3p, miR-574-5p (TARDBP, FUS, C9ORF72 and sporadic mutant Epstein-Barr virus transformed lymphoblastoid cell lines)[168] miR-663a, miR-9-5p (FUS mutant LCLs)[168] Let-7b (FUS and C9ORF72 mutant LCLs)[168]
Huntington's disease	miR-29a, miR-330 (cortex, Brodman area 4)[181] miR-132 (cortex, Brodman area 4)[135] miR-196a[185], miR-486 (cortex, Brodman area 4, only HD1 grade)[135] miR-100, miR-151-3p, miR-16, miR-219-2-3p, miR-219-5p, miR-27b, miR-451, miR-92a (frontal cortex, striatum)[16] miR-10b-5p, miR-1247-5p, miR-196a-5p, miR-196b-5p, and miR-615-3p (prefrontal cortex Brodmann area 9)[189]	miR-132 (cortex, Brodman area 4)[181] miR-9, miR-9*, miR-29b, miR-124a (cortex, Brodman area 4)[135] miR-128a (human pre- and post-symptomatic brain)[184] miR-128, miR139-3p, miR-222, miR-382, miR-433, miR-485-3p (frontal cortex, striatum)[16]	miR-34b (plasma, pre-manifest HD)[166]	

3'UTR = 3'untranslated region; BACE-1 = β -secretase; CNS = central nervous system; CSF = cerebrospinal fluid; ECF = extracellular fluid; HD = Huntington's disease; iPSC = induced pluripotent stem cell; MREs = molecular recognition elements; mRNA = messenger RNA; NEFL = neurofilament light subunit;

miR-153) were shown to regulate APP expression *in vitro* and in cells [41]. Moreover, as shown in cultured cells, some AD-linked mutations in APP interfere with miRNA binding, such as the T117C APP variant, which inhibited miR-147 binding, and the A454G APP variant, which increased miR-20a binding [41]. Alternative splicing of APP is also regulated by miR-124, which is reduced in AD brains leading to APP missplicing [42]. Upregulation of nuclear factor kappa-light-chain-enhancer (NF- κ B)-sensitive inflammatory miRNAs (miR-9, miR-125b, miR-146a, miR-155) was reported in the CSF and extracellular fluid (ECF) from AD neocortex, where A β_{42} levels were slightly reduced, compared with other brain regions, suggesting a role for these miRNAs in the pathogenic signalling of AD [43], potentially disturbing the fine tuning of inflammatory responses as it has been shown that dysregulating only miR-155 or miR-146 is sufficient to trigger autoimmune disease [44–47].

miRNA-mediated regulation of β -secretase 1

Beta-secretase 1 (BACE1) is the proteolytic enzyme that cleaves APP at the β -site APP, resulting in the production of A β . BACE1-targeting miRNAs therefore regulate APP

and A β indirectly. The miR-107 targets BACE1 mRNA and since miR-107 levels significantly decrease during AD progression, BACE1 mRNA levels subsequently increase [37]. Also, miR-195 was shown to regulate negatively BACE1 and A β in cell lines and in the SAMP8 (senescence-accelerated) mouse model, which develops AD signs [48]. Both miR-298 and miR-328 have been shown to regulate BACE1, as their overexpression in cell lines and primary neurons showed lower BACE1 levels [49]. BACE1 expression can be regulated by miR-29a, miR-29b-1, and miR-9 in cultured cells, and the miR-29a/b-1 cluster was significantly decreased in AD brain displaying abnormally high BACE1 protein [50]. Results from cultured cells also showed that miR-124 directly regulates BACE1 expression, with miR-124 downregulation leading to increased BACE1 levels and A β neurotoxicity [51]. Overexpression of miR-29c in cell lines and transgenic mice (APPswe/PS Δ E9) leads to reduced levels of BACE1 [52]. Interestingly, miR-29a is significantly downregulated in human AD brains, leading to the upregulation of its target, neuron navigator 3 (NAV3), in degenerating pyramidal neurons in the cerebral cortex [53].

Misregulation of miRNAs in Alzheimer's disease

In a miRNA microarray screen followed by quantitative polymerase chain reaction (qPCR) validation of hits, Schipper and colleagues demonstrated that miR-34a and miR-181b are significantly upregulated in blood mononuclear cells of patients with sporadic AD, while their expression differed between AD subjects bearing one or two APOE4 alleles [54]. Also, miR-34a was shown to harbour a 3'-untranslated region (3'UTR) binding site on tau mRNA, thereby directly repressing tau expression in cultured cells [55]. Misregulation of miRNAs occurs already in the early stages of AD (Braak III), such as elevation of miR-26b, which leads to apoptosis in primary neurons [56] and decrease of miR-132-3p [57] in postmortem AD brains. Importantly, the latter appears to occur mostly in neurons with tau hyperphosphorylation [57]. In addition to the above, numerous miRNAs were found to be deregulated across different brain areas and AD stages, both in brain and CSF. All members of the miR-30 family were upregulated in CSF while only miR-146b was consistently altered in both hippocampus and medial frontal gyrus in early and late stages of the disease [36]. Moreover, miR-9, miR-125b, miR-146a and miR-155 were upregulated in CSF and ECF of AD patients [43], while miR-384 was downregulated in both serum and CSF of AD patients [38]. Further, miR-9 was found to be upregulated in AD CSF and ECF [43] and hippocampus [58], but downregulated in neocortex [50], also suggesting differential spatial regulation of miRNAs in AD brain. The miRNA signature is also altered in the blood of AD patients. Indeed, blood mononuclear cells of AD subjects showed significantly increased levels of miR-34a and miR-181b [54]. Finally, using nCounter miRNA expression assay v1 (high-throughput expression profiling of miRNAs using digital detection and counting of miRNAs in a single reaction without amplification) followed by qPCR validation, let-7d-5p, let-7g-5p, miR-15b-5p, miR-142-3p, miR-191-5p, miR-301a-3p and miR-545-3p were all shown to be signi-

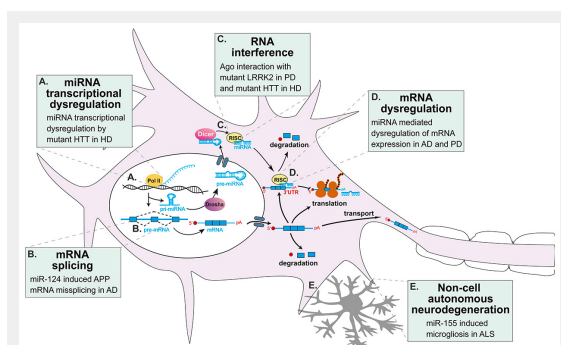


Figure 1

miRNA alterations in neurodegenerative diseases. Numerous alterations in miRNA biogenesis and function were found to contribute to the pathogenesis of several neurodegenerative diseases including: (A) In HD, miRNA transcription is disturbed as mutant huntingtin does not bind REST (which is normally bound to wild-type huntingtin), allowing the formation of NRSE thus repressing the transcription of neuronal miRNAs [135, 181, 183]. Mutant huntingtin also binds to p53 dysregulating p53-mediated transcription of miRNAs [16, 188]. (B) Downregulation of miR-124 has been found to cause missplicing of APP in AD [42]. (C) Ago has been found to be sequestered by mutant huntingtin and accumulated into stress granules in HD suggesting impaired miRNA-mediated mRNA regulation [189–191]. Interestingly, mutant LRRK2 also interacts with Ago, impairing mRNA regulation by miRNAs [72]. (D) Expression of APP and BACE1 in AD [37–41, 48–52] and SNCS and LRRK2 in PD [71–73] has been described to be specifically affected by their regulating miRNA(s) that are disturbed in these diseases. (E) Non-cell autonomous neurodegeneration is now well recognized in ALS and miR-155 seems to play a major role in the pathologic microglial activation [43, 163, 165].

3'UTR = 3' untranslated region; AD = Alzheimer's disease; ALS = amyotrophic lateral sclerosis; APP = amyloid precursor protein; BACE-1 = β -secretase 1; HD = Huntington's disease; HTT = huntingtin gene; LRRK2 = leucine-rich repeat kinase 2; miRNA = microRNA; mRNA = messenger RNA; NRSE = neuron restrictive silencer element; PD = Parkinson's disease; pri-miRNA = primary miRNA; REST = RE1-silencing transcription factor; RISC = RNA-induced silencing complex

ificantly upregulated in plasma of AD patients, providing differentiation of AD patients from normal controls with >95% accuracy [59].

Taken together, the above results clearly demonstrate the high potential of miRNAs as biomarkers for AD. Discrepancies between studies, however, indicate that additional multicentre in-depth studies are necessary to identify and/or confirm miRNAs that could represent reliable biomarkers for AD.

Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder, which affects an estimated 1% of individuals over 60 years of age, a percentage that is expected to double by 2030 as the population ages [60]. PD is a progressive neurodegenerative disease presenting with resting tremor, bradykinesia, rigidity, postural instability and dementia [61]. These symptoms are attributed to loss of dopamine neurons in the substantia nigra pars compacta, as well as widespread neuropathological changes in multiple brain regions, including the amygdala, cingulate gyrus and cerebral cortex [61]. The pathological hallmark of PD are Lewy bodies, intraneuronal inclusions that mainly contain aggregated α -synuclein, often in association with neurofilaments and ubiquitin [62]. It has been shown that expression of mutant as well as overexpression of wild-type α -synuclein leads to development of PD [63]. PD is mainly sporadic and is believed to develop as a consequence of multiple genetic and possibly environmental factors in an aging brain. Mutations in approximately 15 genes were reported to cause rare cases of familial PD, including mutations in α -synuclein, LRRK2, parkin, PINK1, DJ-1 [64]. Interestingly, it is well-recognised that temporary Parkinsonian symptoms represent a common side effect of several drugs that usually vanishes after drug withdrawal [65]. However, specific cases of irreversible Parkinsonism were described in the late 70s and early 80s among drug abusers in the USA. The chemical compound responsible for the PD-like symptoms and severe neuronal loss in the substantia nigra was later identified to be MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), the side product in synthesis of the 'new synthetic heroin', MPPP (1-methyl-4-phenyl-4-propionoxypiperidine) [65–67]. Subsequently, MPTP became one of the most commonly used neurotoxins to model PD in experimental animals [68].

miRNAs were first suggested to be involved in PD pathology by deletion of Dicer specifically in mouse dopaminergic neurons, which resulted in their loss and consequently in reduced locomotion [69]. Levels of miR-133b are significantly decreased in the midbrain of PD patients compared with controls, leading to increased levels of the transcription factor Pitx3, which in turn specifically induces transcription of miR-133b in a negative feedback loop that is important for neurogenesis and function of dopaminergic neurons [69]. Later studies revealed that miRNAs are directly involved in PD pathogenesis, regulating pathways of PD-linked genes and their protein products. Through use of cultured cells, brain enriched miR-7 [70] and miR-153 [71] have been shown to inhibit SNCA mRNA and conse-

quently α -synuclein protein targeting the 3'UTR of SNCA, suggesting that upregulation of miR-7 and miR-153 may represent a viable therapy for PD [71]. Mutant leucine-rich repeat kinase-2 (LRRK2) inhibits let-7 and miR-184* function leading to upregulation of their targets, E2F1 and DP, respectively, ultimately causing LRRK2-linked dopaminergic neuron pathogenesis in *Drosophila* [72]. Importantly, mutant LRRK2 has been shown to interact with and regulate argonaute protein in both *Drosophila* brains and cultured cells [72], suggesting global miRNA deregulation. Moreover, miR-205 has recently been shown to be downregulated in the brains of sporadic PD patients, with increased LRRK2 levels [73]. Since miR-205 directly regulates LRRK2 mRNA and protein, downregulation of miR-205 may contribute to pathogenic elevation of LRRK2 and, therefore, overexpression of miR-205 may represent potential therapy for PD [73]. SNPs in the 3'UTR of FGF20 were shown to be regulated by miR-433 in both cultured cells and in PD brains [74] and are associated with elevated levels of both FGF20 and α -synuclein, which has been linked to increased risk of developing PD [74, 75], but the association between FGF20 and PD remains controversial [76, 77].

Circulating miRNAs in peripheral blood were shown to differentiate control individuals from untreated PD patients (miR-1, miR-22, miR-29) and untreated from treated PD patients (miR-16-2, miR-26a2, miR30a) [78]. However, a later study with a substantially larger cohort of plasma samples did not confirm those results and instead showed only miR-331-5p to be significantly upregulated in PD patients [79]. Clearly, differences in sample types and patient cohorts are most likely the reason for these variations and more comprehensive studies are needed to identify the right miRNAs that are present across different PD types. Nevertheless, these results are encouraging and represent a huge step towards easily accessible and reliable biomarkers of PD.

Prion diseases

Prion diseases, also known as transmissible spongiform encephalopathies, are a group of rare fatal neurodegenerative disorders marked by neuronal loss, gliosis and spongiform changes in the brain. Prion diseases include Creutzfeldt-

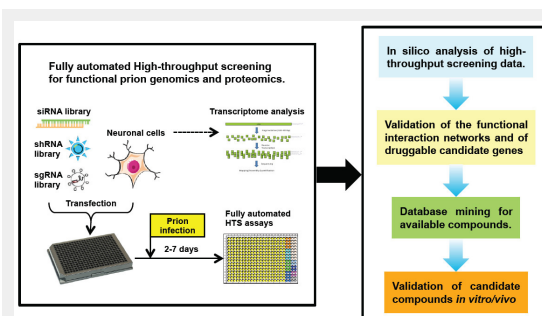


Figure 2

Proposed new strategies for high throughput small RNA screens to discover cell signalling mechanisms and novel therapeutic target for prion disease.

HTS = high throughput screening; sgRNA = single guide RNA; shRNA = short hairpin RNA; siRNA = small interfering RNA

Jakob disease in humans, scrapie in sheep and goats, chronic wasting disease in deer and elk, and the bovine spongiform encephalopathy in cattle [80]. The disease-causing agent consists of PrP^{Sc}, which is an isoform of the host-encoded cellular prion protein PrP^C. PrP^{Sc} acts as a propagator that is capable of seeding a self-perpetuating reaction to convert PrP^C to the pathological conformation of PrP^{Sc} within a bioassay system [80], a process termed prion replication.

The expression of PrP^C is indispensable for prion replication and prion-induced neurodegeneration. However, the intrinsic mechanisms underlying prion replication and subsequent neurodegeneration are largely unknown [81]. Several key questions, including the genes and proteins that regulate endogenous PrP^C expression, the machinery of PrP^C to PrP^{Sc} conversion, and the molecules affecting susceptibility to prion infection and cell-to-cell spread of prions, have not been answered yet. This is mainly because of the dearth of high-throughput technologies that can be used to identify these mechanisms, which would not only greatly improve our basic understanding of prion pathophysiology, but might also uncover novel therapeutic and diagnostic targets. Here we discuss the application of small RNA-based technologies in prion studies.

RNA profile change in neuronal cells upon prion infection

Upon prion infection of neuronal cell lines, the transcriptome profile changes reflect the direct response to prions and could indicate potential pathways leading to prion pathogenesis. Greenwood et al. performed the first study using a complementary DNA (cDNA) microarray and observed strikingly different expression profiles in both ScN2a and ScGT1 cells upon persistent prion infection [82]. Interestingly, many of the differentially regulated genes were also observed to change in other neurodegenerative disorders, suggesting some conserved responses to neurological damage in various neurodegenerative conditions. However, another transcriptional study conducted by Julius et al. analysed the transcriptome of several prion-infected cell lines (N2aPK1, CAD and GT1) under more stringently controlled conditions. By applying high-density oligonucleotide microarray analysis and exhaustive bioinformatical interrogation of the data, Julius et al. identified very modest differential expression resulting from prion infection [83]. The discrepancy between the two studies may be due to differences in experimental design, cell culture conditions and/or prion infection processes. Among many possible factors, the clonal segregation strategy used in the Greenwood study might have resulted in differential transcriptional responses independent of prion infection.

The transcriptional profile of cell culture might change after prion infection; likewise, the transcriptome of cell lines could also affect the susceptibility to prion infection. Different cell lines have distinct susceptibilities to prion infection. These differences cannot be explained only by the PrP^C expression levels, which are required but not sufficient for prion replication [84]. Recently, Marbiah et al. isolated prion-resistant revertants from susceptible cells. By comparing the transcriptome of prion-resistant revertants to that of susceptible cells, Marbiah et al. revealed a

gene regulatory network for extracellular matrix remodeling that is associated with prion propagation.

The roles of miRNAs in prion diseases remain unclear. By using microarray and real-time PCR (RT-PCR), Saba et al. profiled miRNA expression changes in the brains of mice intracerebrally inoculated with mouse scrapie. Fifteen miRNAs were found to be deregulated during the disease processes and only one of them had previously been shown to be deregulated in neurodegenerative disease [85], suggesting a prion disease-specific pattern of differentially expressed miRNAs. In addition, a correlation between miRNA expression profile and expression change on putative gene targets was identified. Further studies are required to determine the cell types in which miRNAs are deregulated.

In conclusion, with proper experimental strategies and application of new RNA analysis technologies such as RNA sequencing, we expect that our understanding of the cellular transcriptional response to prion infection will be deepened. The identification of RNA profile changes upon prion infection may have potential use as biomarkers for prion diagnostics and therapeutic targets.

Small RNA-based target-identification and potential therapeutics for prion disease

PrP^C is indispensable for prion replication and prion-induced pathogenesis. Mice devoid of PrP^C showed resistance to prion infection [86]. PrP^C reduction of 50% (*Prnp*^{+/-} heterozygous mice) significantly prolongs the incubation time of prion disease in mouse, while overexpression of PrP^C markedly shortens prion disease progression [87]. Therefore, identification of genes involved in PrP^C biosynthesis by use of small RNA-based screening and RNA-mediated modulation of PrP^C expression accordingly have great potential to mitigate the PrP^C-PrP^{Sc} conversion, diminish prion-induced neurotoxicity and consequently slow disease progression.

Since *Prnp* is the main determinant for prion pathogenesis, small RNAs specifically and directly targeting *Prnp* have been extensively studied for prion therapeutics both *in vitro* and *in vivo*. Small interfering RNA duplexes targeting the *Prnp* gene effectively suppressed expression of PrP^C in prion-infected N2a cells and inhibited PrP^{Sc} accumulation [88]. A liposome-siRNA-peptide complex was later developed to suppress PrP^C expression and eliminate PrP^{Sc} formation in prion-infected N2a cells [89]. Additionally, the vector-based short hairpin RNA (shRNA) was successfully applied to decrease PrP^C expression in rabbit kidney epithelial cells [90] and mouse N2a cells [91]. Lentiviral vector delivered shRNA also decreased PrP^C levels and extended the prion incubation time in mice [91, 92]. Similar effects were observed in transgenic livestock [93, 94]. Therefore, RNA interference (RNAi) based *Prnp* knock-down represents a promising approach for prion therapeutics. However, there are concerns about RNAi-based therapeutics, such as immunogenicity and off-target induced cytotoxicity. Hence, modified versions of small RNAs or novel small RNAs targeting *Prnp* may circumvent those pitfalls. Recently, miRNA [95], DNA-based antisense oligonucleotides [96], and RNA aptamers [97, 98] targeting

Prnp/PrP^C have been shown to prevent prion disease progression.

HTS assays including PrP-FEHTA (PrP-FRET-enabled high throughput assay) [99] and ELISA (enzyme-linked immunosorbent assay) [100] have been applied to search for small compounds that may reduce PrP^C expression for prion therapy, albeit no strong candidates have been discovered yet. An alternative strategy is RNAi screening, which is based on RNA-mediated gene silencing that can reduce levels of the mRNA target [101]. RNAi HTS has become a powerful tool for whole transcriptome manipulation and thus discovery of signalling mechanisms and therapeutic strategies in human diseases [101]. Transcriptome wide RNAi screenings (siRNA or shRNA libraries) are likely to uncover genes and proteins that are involved in PrP^C biosynthesis. Moreover, the advent of CRISPR-Cas9 mediated single guide RNA (sgRNA) library HTS provides another alternative approach to interrogation of the molecular networks that regulate PrP^C biosynthesis. Hits identified by those RNA-based HTS represent potential targets for modulating PrP^C expression and therefore therapeutics for prion disease.

In addition, RNA-based HTS can be applied to identify genes and proteins that are involved in prion replication. However, this application is challenging because the conventional quantification of prion infectivity is performed by titration of serial dilutions using mouse bioassays [102] or standard scrapie cell endpoint assays [103]. Both approaches are either protracted or demanding substantial manual work, hence are not applicable for HTS. Therefore, new methodologies and technologies are required to meet the need of a reliable prion infectivity assay complementary to an automated high-throughput system, which will facilitate HTS of entire small RNA libraries including siRNA, shRNA or the newly developed sgRNA libraries, as well as compounds and peptides, with the aim of identifying disease relevant genes, early molecular markers and novel therapeutics (fig. 2). The progress of small RNA-based HTS technology is exciting and may contribute to the discovery of promising therapeutic targets for prion diseases and other neurodegenerative diseases.

Amyotrophic lateral sclerosis and frontotemporal dementia

Amyotrophic lateral sclerosis (ALS; also known as Lou Gehrig's disease [USA] and motor neuron disease [UK]) is a late onset, progressive and fatal neurodegenerative disease characterised by loss of upper motor neurons in the motor cortex and lower motor neurons in the brain stem and spinal cord. Motor neuron neurodegeneration in ALS leads to muscle atrophy, weakness, fasciculation and spasticity, followed by paralysis and ultimately death, typically due to respiratory failure [104]. First described in 1869 by Jean-Martin Charcot (therefore initially known as a Charcot's disease), ALS was named based on his observations of distinct loss of axons in the lateral aspect of the spinal cord [105]. Typical onset of the disease is usually in mid-life (45–60 years) with an uncompromisingly progressive disease course of 3 to 5 years [106]. Worldwide incidence and prevalence of ALS are 1–2 and 4–6 per 100 000

each year, respectively, with a lifetime ALS risk of 1/350 to 1/1 000 [107, 108] and incidence peak (10–15/100 000/year) between ages 60 and 79 [109]. The vast majority of ALS cases (~90%) are sporadic with no family history. Sporadic ALS includes patients carrying *de novo* mutations or alleles with incomplete penetrance, or with an incomplete family history. The remaining 10% of ALS cases are caused by autosomal dominant mutations and therefore called familial ALS. Approximately 20% of familial ALS cases are associated with mutations in a gene coding for an antioxidative enzyme, the copper/zinc superoxide dismutase 1 (SOD1) [110]. Recent identification of causative mutations in the 43-kDa transactive response (TAR) DNA-binding protein (TDP-43) [111–5] and fused in sarcoma/translocated in sarcoma (FUS/TLS) [116, 117], which are both involved in RNA processing, in conjunction with the recognition that TDP-43 is the main protein component of the ubiquitinated inclusions in the vast majority of ALS patients [118, 119], led to the idea that aberrant RNA metabolism contributes to ALS pathogenesis [106, 120, 121]. More recently, hexanucleotide expansions in an intronic region of a poorly characterised gene called *C9ORF72* (chromosome 9 open reading frame 72) were identified as the most common genetic cause of ALS [122–4]. This finding corroborated the link between ALS and frontotemporal dementia (FTD), which are now recognised as two clinical ends of one disease spectrum [106, 125, 126]. Indeed, it is estimated that about 20% of ALS patients develop signs of frontotemporal dementia [127] and that about 15% of FTD patients develop motor neuron degeneration [126, 127].

FTD is the clinical presentation of frontotemporal lobar degeneration (FTLD), which is a neurodegenerative disease characterised by progressive neuronal loss, predominantly involving the frontal and temporal lobes [128], that primarily affects behaviour, social awareness and language [129]. FTD has only recently been appreciated as one of the leading causes of dementia, particularly in patients younger than 65 years [130], making it the second most common cause of early onset dementia after Alzheimer's disease [131]. Data from the UK show a prevalence of around 15 cases per 100 000 population aged 45–64 years [131]. Approximately 50% of all FTD cases are familial with the microtubule-associated protein tau (MAPT) and progranulin (PGRN) accounting for 10%–20% of FTD cases [126]. Rare cases of FTD are caused by mutations in TDP-43 and FUS/TLS [120] but the strongest genetic link between ALS and FTD is the hexanucleotide expansion in *C9ORF72* which is the most common genetic cause of FTD-ALS and accounts for a large number of FTD cases (10%–30%) [122–4, 126].

miRNA miR-9 in (motor) neurons

miRNA is essential for normal function of spinal motor neurons as ablation of *Dicer1* in motor neurons induced denervation, muscle atrophy, astrogliosis and motor neuronal loss [132]. Deletion of *Dicer* in astrocytes led to significant downregulation of astrocyte-specific glutamate transporters GLT-1 and GLAST resulting in non-cell autonomous neuronal dysfunction and degeneration, demonstrating its crucial role in these glial cells [133]. Interestingly, in a mouse model of a loss of miRNA function

in spinal motor neurons (MNDicer^{MUT} mice), miRNA-deficient spinal motor neurons showed specific upregulation of the neurofilament heavy subunit (NEFH) that has been previously linked to motor neuron degeneration. Coordinated neurofilament gene expression is necessary for normal neuronal function and upregulation of only NEFH in the motor neurons of MNDicer^{MUT} mice, suggests possible perturbation in the fine-tuning of related genes by miRNAs. Indeed, authors found a single miR-9-binding site on the neurofilament light subunit (NEFL) mRNA and nine different sites in the NEFH mRNA. Furthermore, motor neurons differentiated from mouse embryonic stem cells harbouring an SMN1 mutation revealed a 15-fold decrease in the expression of both miR-9 and miR-9* [132]. Importantly, human ALS neurons derived from induced pluripotent stem cells (iPSCs) (TARDBP A90V mutation) also displayed downregulation of miR-9 and miR-9-2 [134], which may be a common mechanism in several neurodegenerative diseases, including ALS, FTD, spinal muscular atrophy and Huntington's disease. Indeed, miR-9 and miR-9* have been shown to regulate directly RE1-silencing transcription factor (REST) and CoREST, respectively, which could lead to increased or mislocalised pools of REST thereby dysregulating neuronal genes [135]. In addition, miR-9, among others, was also found to be significantly downregulated in the spinal cord of human sporadic ALS patients [136]. However, this study describes NEFL mRNA suppression by dysregulated miR-146a*, miR-524-5p and miR-582-3p, which are capable of direct binding to NEFL mRNA [136]. Moreover, the same group reported that additional miRNAs that normally stabilise NEFL mRNA, miR-b1336 and miR-b2403, were downregulated in ALS spinal cord, thereby contributing to the loss of NEFL steady-state levels [137]. In contrast to the findings in human ALS samples, miR-9 expression was upregulated in the spinal cord of SOD1^{G93A} mice at early and late symptomatic stages [138], suggesting that this mechanism may vary among different types of ALS with distinct pathologies (primarily TDP-43 pathology in sporadic ALS or TARDBP A90V mutation carriers, versus misfolded SOD1 in the SOD1^{G93A} mice).

TDP-43 and FUS miRNA regulation

The physiological role of the ALS/FTD-linked RNA-binding proteins TDP-43 and FUS has been extensively studied, which showed that they are involved in many aspects of RNA metabolism (reviewed in [120, 121, 126]), including miRNA regulation. Indeed, TDP-43 was shown to associate with Drosha [139] and Dicer complexes [140, 141], an interaction that may be disturbed by ALS-linked mutations that increase the protein half-life of TDP-43 [139], thereby interfering with miRNA biogenesis. In addition, FUS interaction is required for efficient recruitment of Drosha at specific pri-miRNA sites at early stages of transcription thereby stimulating miRNA biogenesis [149].

TDP-43 knockout in cultured cells led to downregulation of let-7b and upregulation of miR-663 expression [142]. Fan and colleagues recently applied transcriptome-wide analysis of TDP-43 binding small RNAs on previously published CLIP-seq data from human and mouse samples [144, 145]. Their analysis identified a novel NRXN1

intron-derived miRNA, called miR-NID1, found in both human and mouse [143]. This miRNA is located in the fifth intron of human neurexin 1 (*NRXN1*) and four pre-miR-NID1 candidates are located in the introns of protein coding genes in the mouse. Functional analysis by means of nucleocytoplasmic fractionation and RNA immunoprecipitation assays in SY-5YC cells revealed that endogenous TDP-43 is associated with miR-NID1 in both cytoplasm and nuclei and that miR-NID1 represses the expression of NRXN1 through TDP-43 [143].

Overexpression of human wild type FUS in transgenic mouse models has been shown to induce progressive motor neuron degeneration [146] and its deficiency resulted in chromosomal instability and perinatal death [147]. FUS carrying a 3'UTR G48A mutation, which affects the binding site of miR-141/200a, renders *FUS* mRNA insensitive to repression by these miRNAs [148]. Furthermore, as shown in SK-N-BE neuroblastoma cells, FUS controls miR-141/200a biogenesis suggesting a feed-forward regulatory loop in which FUS increases the expression levels of two miRNAs, which in turn regulate FUS accumulation and an increase in nuclear FUS protein [148]. Moreover, expression of a subset of miRNAs known to have a crucial role in neuronal function, differentiation and synaptogenesis (miR-9, miR-125b and miR-132), is altered upon FUS knockdown in cultured cells [149].

Expression of miR-206 in ALS

The importance of miR-206 expression in ALS was first highlighted by a study on neuromuscular junctions of mutant SOD1 mice [150]. The investigators found that skeletal muscle-specific miR-206 is dramatically increased in the SOD1^{G93A} mouse model of ALS. In this study, they generated miR-206 knockout mice, which were also crossed with transgenic mice expressing low levels of SOD1^{G93A}. The miR-206 knockout mice developed normally but the SOD1^{G93A} mice lacking miR-206 displayed exacerbation of disease symptoms accompanied by accelerated atrophy of skeletal muscle, leading to kyphosis, paralysis and death [150]. It has been shown that axonal branches of surviving motor neurons are partially reinnervating muscles whose innervating motor neurons have already died during the course of ALS. Also, miR-206 slows ALS progression by sensing motor neuron injury and promoting the compensatory regeneration of neuromuscular synapses via induced secretion of fibroblast growth factor binding protein 1 (FGFBP1) from muscle by inhibiting HDAC4 translation [150]. This has been confirmed in patients as miR-23a, miR-29b, miR-206 and miR-455 were all increased in skeletal muscle of ALS patients compared with healthy controls [151]. It was found that miR-23a repressed PGC-1 α translation in a 3'UTR dependent manner *in vitro* and that transgenic mice overexpressing miR-23a had a reduction in PGC-1 α , cytochrome-b and COXIV protein levels leading to skeletal muscle mitochondrial dysfunction in ALS [151]. Recently, miR-206 was found to be consistently increased in fast-twitch muscles during the course of the disease in SOD1^{G93A} mice, with highest expression levels towards the end stage [152]. Most importantly, miR-206 was also found to be significantly el-

evated in circulating blood of both SOD1^{G93A} mice and human ALS patients [152].

Dysregulation of miRNAs in frontotemporal dementia

FTD and ALS are linked clinically, pathologically and molecularly by several lines of evidence and thus represent two ends of a disease spectrum [153]; therefore, a common miRNA biogenesis dysregulation by mutant TDP-43 and FUS can be expected. Nevertheless, progranulin (*GRN*) mutations are exclusively found to cause FTD and loss of progranulin function causes tau negative FTD with TDP-43 and ubiquitin pathology (FTD-TDP) [154, 155]. Moreover, a common genetic variant (rs5848), located in the 3'UTR of *GRN* within a binding-site for miR-659, is a major susceptibility factor for this type of FTD, as miR-659 binds the high-risk T allele of rs5848 more efficiently, resulting in enhanced translational inhibition of *GRN* [156]. Using miRNA array profiling and qPCR validation in both frontal cortex and cerebellum of FTD-TDP patients carrying *GRN* mutations, the same group found that miR-922, miR-516a-3p, miR-571, miR-548b-5p, and miR-548c-5p are exclusively dysregulated in these patients when compared with FTD-TDP patients without *GRN* mutations [157]. A genome-wide association study showed that chromosome 7p21 variants within the gene *TMEM106B* confer increased risk of developing FTD-TDP [158]. The same group later found that *TMEM106B* is repressed by miR-132 and miR-212 and that both miRNAs are downregulated in FTD-TDP leading to aberrant overexpression of *TMEM106B* that affects the distribution and intracellular levels of progranulin, suggesting that the two proteins may act in the same pathogenic pathway in FTD-TDP [159]. Specific SNPs in the *TMEM106B* gene seem to regulate progranulin levels and thereby the penetrance of FTD in *GRN* mutation carriers [160]. Recently, *TMEM106B* has been identified as the first genetic factor modifying disease presentation in *C9ORF72* expansion carriers, as homozygosity for the minor rs3173615 allele protects carriers from developing FTD, but not from developing ALS [161]. Furthermore, a SNP that confers increased risk for developing FTD-TDP (major, or T, allele of rs1990622) and which is associated with later age at onset and death in *C9ORF72* expansion carriers has been also identified [162]. Most recently, a genotype that confers decreased risk for developing FTD-TDP (minor, or C, allele of rs1990622) has been found to be associated with earlier age at onset and death in *C9ORF72* expansion carriers [134] completing the picture of *TMEM106B* as a genetic modifier in ALS and FTD.

miRNA overexpression or inhibition as a potential therapy in ALS/FTD

The levels of miR-206 are significantly elevated in muscles [150] and plasma [152] of SOD1^{G93A} mice, and in the plasma of sporadic ALS patients [152]. Since miR-206 senses damage in motor neurons and promotes compensatory mechanisms that lead to regeneration of neuromuscular junctions, its increased expression acts to counteract disease progression in the SOD1^{G93A} mouse model, but ultimately fails to completely reverse disease [150]. Nevertheless, it is possible that overexpression of miR-206 in skeletal muscles could further delay the muscle weakness

observed in ALS patients, thereby providing a potential palliation approach.

Recent microarray screens revealed that miR-155 is significantly upregulated in SOD1^{G93A} mouse and human ALS patient spinal cords [163], as well as in peripheral monocytes from ALS patients [164]. Furthermore, downregulation of miR-155 in the CNS of SOD1^{G93A} mice using the antisense oligonucleotide anti-miR-155 resulted in significant extension of survival by 10 days and disease duration by 15 days (38%) in comparison with scrambled oligonucleotide control-treated mice [163]. Given the important proinflammatory function of miR-155 [44–7] and the fact that miR-155 has been found to be upregulated in AD CSF and neocortex ECF [43], it is possible that the elevated miR-155 levels in ALS reflect the ongoing neuroinflammation. As the administration of anti-miR-155 caused global derepression of targets in peritoneal macrophages after intraperitoneal treatment with lipopolysaccharide in mice, and since the anti-sense oligonucleotides were detected in CNS microglia upon intraventricular administration [163], the significant extension of survival of SOD1^{G93A} mice may be associated with reduced non-cell autonomous neuronal toxicity of glial cells [165]. As miR-23a, which is overexpressed in the skeletal muscle of ALS patients, was found to repress PGC-1 α translation in a 3'UTR dependent manner both *in vitro* and in mice, leading to skeletal muscle mitochondrial dysfunction in ALS [151], inhibition of miR-23a in skeletal muscle may represent potential therapy in ALS.

miRNAs as biomarkers in ALS/FTD

Microarray screening of 911 human miRNAs in leucocytes obtained from 8 sporadic ALS patients and 12 healthy controls revealed that miR-451, miR-1275, miR-328-5p, miR-638, miR-149 and miR-665 were downregulated across different gender groups, as well as in all examined samples. Importantly, qPCR analysis of miR-338-3p in 14 sporadic ALS patients and 14 controls confirmed the specific miR-338-3p upregulation in leucocytes of all ALS patients, suggesting that this miRNA could represent an invaluable marker for ALS that could offer early diagnosis through a blood test [167].

A qPCR screen of TDP-43 binding miRNAs in human serum and CSF found that out of the 9 recently discovered TDP-43 binding miRNAs, miR-132-5p, miR-132-3p and miR-143-3p were significantly downregulated, and miR-143-5p and miR-574-5p were significantly upregulated in serum and CSF of matched sporadic ALS patients suggesting a systemic epigenetic dysregulation in ALS [168].

Huntington's disease

Huntington's disease (HD) is a fatal inherited autosomal dominant neurodegenerative disorder caused by an expansion of the CAG repeat localised in the first exon of the huntingtin gene (*HTT* or *IT-15*) [169]. Expression of the mutant huntingtin protein with an expanded poly-glutamine tract [169] induces progressive neurodegenerative changes in the whole brain, with the main pathology observed in the striatum, cortex [170] and white matter [171,

[172], consequently leading to progressive motor dysfunction, cognitive decline and psychiatric disturbance [173]. Prevalence of the mutation/disease is about 4–10 per 100 000 in populations of Western European and North American descent, with many more at risk of the disease [170, 174]. CAG repeats longer than 40 are associated with practically full penetrance by the age of 65 years [173, 175]. Longer CAG repeats predict earlier onset of the disease [175], accounting for up to 50%–70% of variance in age of onset [173] with the remaining variance most likely to be attributed to modifying genes (40% of remainder) and the environment (60% of remainder) [176].

Most HD patients have expansions ranging from 40–55 CAG repeats [170, 175], leading to the onset of the disease symptoms in middle age (40 years in average), whereas juvenile onset (under the age of 20) is associated with CAG repeats of 60 or longer [175]. The disease progresses over 15–20 years from onset and culminates in death [177].

REST-induced mRNA and miRNA transcriptome disruption in Huntington's disease

It has long been known that the mutant huntingtin disrupts transcription as wild-type huntingtin sequesters the available REST/NRSF (RE1-silencing transcription factor; aka neuronal restrictive silencing factor) in the cytoplasm preventing it from forming the nuclear co-repressor complex at the RE1/NRSE (repressor element 1; aka neuron restrictive silencer element) nuclear site and allowing gene transcription of neuronal genes, while the mutant huntingtin does not [178]. It was therefore hypothesised that in the CNS, huntingtin may represent a general coordinator of neuronal gene transcription [178, 179]. Further research revealed that REST transcriptionally represses the miRNA family that includes miR-124a, one of the most common and highly expressed CNS miRNAs. [180]. During neuronal differentiation, similar to canonical REST-regulated genes, miR-124a expression is only allowed when REST dissociates from its chromatin binding sites. Diminishing miR-124a activity in mature neurons resulted in specific increase of non-neuronal genes, further demonstrating the neuronal specificity of miR-124a [180].

REST-regulated miRNAs in HD thus became widely researched. In the pivotal search for miRNA dysregulation in HD, REST binding sites [181] were compared with the positions of known miRNA genes [182], which identified 17 possible miRNA targets of REST, including 13 mouse orthologous miRNA-RE1 pairs [181]. Chromatin immunoprecipitation assay confirmed that the identified miRNA RE1s are indeed capable of interaction with REST both *in vitro* and *in vivo*. MiR-132 was found to be downregulated in brains of both human HD patients and R6/2 mice, the most commonly used model of HD expressing the human *HTT* exon 1 with 144 CAG repeats under the control of the human *HTT* promoter. Other miRNAs, however, showed divergent results, such as miR-29a, which is significantly upregulated in HD patients, but downregulated in R6/2 mice and miR-330, which was significantly upregulated in HD samples, but undetectable in R6/2 mice [181]. These data demonstrate that apparent differences could be expected across the samples of different origin, i.e. human HD and animal HD models that apparent differences could

be expected across the samples of different origin (i.e., human HD patients and animal HD models).

Changes of miRNA expression levels correspond to target gene expression in Huntington's disease and correlate with disease progression

Nevertheless, further research brought significant functional discoveries of how miRNAs are involved in the pathogenesis of HD. REST-regulated miRNAs miR-9, miR-9*, miR-29b, miR-124a (all downregulated), and miR-132 (upregulated) differ significantly with increasing HD grade as detected with qPCR of human brain samples from Brodmann area 4 [135]. Both miR-196a (six-fold) and miR-486 (three-fold) were also found to be significantly upregulated in HD1 grade samples. Importantly, this study revealed that miR-9 targets REST and miR-9* targets CoREST, the components of the REST repressor complex [135]. The authors explained the conflict of their results with those of Johnson et al. [181] by the fact that while they analysed mature miRNAs, Johnson et al. used precursor miRNAs [135]. In an *in-vitro* study comparing the miRNAs expression in HdH^{109/109} and HdH^{7/7} cells with qPCR, Soldati et al. [183] confirmed that many HD-dysregulated miRNAs are directly repressed by elevated levels of REST, including miR-9, miR-9*, miR-29b, miR-124, miR-132, miR-135b which is consistent with previous findings [135, 181], but also previously not reported miR-137 and miR-153, as well as REST-indirectly controlled miR-222. Most importantly, they confirmed that impairment in the widespread regulation of miRNA expression by REST results in increased gene expression of miRNA targets in HD as they show that, for instance, Ak2, Elov1 and Ctdsp2 (miR-124 targets), are upregulated in HdH^{109/109} cells [183].

Similarly, in one of the best large animal models for HD, the transgenic HD monkey (rhesus macaque), as well as in human HD brains, miR-128a was found to be significantly downregulated. It has been shown with a luciferase assay that miR-128a regulates the HD canonical signalling genes HIP-1, HTT, SP-1 and GRM5 [184]. Microarray analysis of 352 rhesus miRNAs led to detection of 11 dysregulated miRNAs in the frontal cortex. Four miRNAs with significant target association to the HD canonical pathway were further analysed with qPCR, which validated significant downregulation of miR-181c, miR-128a and miR-133c, and significant upregulation of miR-194 [184]. The same group has also previously discovered that miR-196a indirectly regulates the expression and aggregation of mutant huntingtin both *in vitro* and *in vivo*, involving miR-196a-induced alteration of ubiquitin-proteasome system, gliosis, and the cyclic AMP response element-binding protein (CREB) pathway [185].

Regulation of miRNA by p53 in Huntington's disease

Using frontal cortex and striatal samples isolated from HD patient brains and appropriate controls, REST-regulated, as well as REST-independent miRNAs, were found to be dysregulated by means of next generation sequencing [16]. The latter approach revealed length and sequence heterogeneity for almost all miRNAs (isomiRs) and that the mechanisms governing the expression of identified miRNAs and their corresponding isomiRs are mostly par-

allel. Importantly, it was also discovered that the promoter region of the downregulated miRNAs in frontal cortex harbour significantly more p53 regulatory motifs [16] and p53 was previously shown to regulate multiple miRNAs [186]. Mutant huntingtin binds P53 and induces its overexpression [187]. In addition, p53 regulates most miRNAs among all transcription factors studied and this can be explained by its overexpression in HD [188]. Therefore, p53 may contribute to the widespread downregulation of miRNA in HD [16, 188].

Chromosomal clusters display similar regulation in Huntington's disease

Significant upregulation of five miRNAs (miR-10b-5p, miR-196a-5p, miR-196b-5p, miR-615-3p and miR-1247-5p) has been identified with next generation sequencing of samples from HD and normal control brains (prefrontal cortex [Brodmann area 9]) and the differential expression of these miRNAs was further validated with qPCR. Interestingly, miR-10b-5p, miR-196a-5p, miR-196b-5p and miR-615-3p are strongly related to Hox cluster genes [189]. Moreover, it was found that miRNA in chromosomal clusters exhibited similar expression patterns in HD; for example, let-7a, let-7c, let-7d and let-7e were all downregulated in HD frontal cortex and striatum and miR-30a, miR-30b, miR-30c and miR-30e were upregulated in the same tissues [16]. Additionally, miR-10b-5p, miR-196a-5p, miR-196b-5p and miR-615-3p, which are miRNAs strongly related to Hox cluster genes, were all found to be significantly upregulated in prefrontal cortex of HD patients [189]. Lastly, expression of IsomiRs and the corresponding miRNAs are in most cases parallel [16].

miRNA regulation by stress granules

The interesting hypothesis that mutant huntingtin impairs miRNA-mediated mRNA regulation and local translation, via its accumulation into stress granules together with argonaute 2, an RNA-induced silencing complex (RISC) component, which is required for RNA-mediated gene silencing, has been proposed [189–91]. TDP-43 and FUS accumulate into stress granules as a normal physiological response to stress [192, 193] and it has been hypothesised that the association of TDP-43 and FUS/TLS with stress granules may be an initiating event which, following chronic stress, eventually leads to irreversible pathological aggregation [126]. Taken together, the above highlight the possibility that pathological accumulation followed by co-aggregation of these proteins with RISC and/or Drosha and Dicer complexes and multiple mRNAs could represent a common pathological mechanism in both ALS/FTD spectrum diseases and HD.

Dysregulation of miR-196a associates with clinical features of Huntington's disease and represents potential therapeutic agent in Huntington's disease

Next generation sequencing data of a relatively large number of patient samples allowed association studies of up-regulated miRNAs with clinical features of HD. It was discovered that CAG repeat size correlates with miR-10b-5p and miR-196a-5p, age of motor dysfunction onset with miR-10b-5p, miR-196a-5p and miR-196b-5p, and age at

death with miR-10b-5p and miR-615-3p [189]. Previously, miR-196a had been found to be significantly overexpressed in HD1 brains [135] and in mutant huntingtin transfected NT2-derived neurons [166], and its overexpression resulted in downregulation of mutant huntingtin in human iPSC-derived HD neurons and in an HD mouse model [185]. It is, therefore, possible that increased miR-196a expression is an adaptive response promoting neuronal survival [189] but that its endogenous activation in HD is not sufficient to ameliorate the HD pathology [185]. However, overexpression of miR-196a may represent a potential therapeutic approach [185, 189].

miRNAs as biomarkers in HD

In order for miRNAs to become valuable biomarkers of HD onset and/or progression, they obviously need to be detectable and adequately stable. Importantly, human plasma miRNAs were reported to withstand repeated freeze-thaw cycles and, in only small volumes of plasma, p53-regulated miR-34b was found to be elevated in premanifest HD patients as detected with qPCR [166]. An earlier study of HD patient-derived whole blood identified multiple significantly upregulated mRNAs compared with controls [194], but this finding could not be replicated [195]. Next generation sequencing of plasma samples with further qPCR validation of miR-34b expression obtained from a large HD patient cohort is therefore absolutely necessary.

miRNAs in muscle dystrophy

The first evidence for an important role of miRNAs in mammalian myogenesis was shown for the paradigm of myoblast to myotube conversion [196–8]. Subsequently, there was an increasing interest for the regulation of miRNAs in muscle diseases. Muscular dystrophies are a heterogeneous class of >30 different inherited myopathies [199] that were an obvious start for this search. They show a high cycling of muscle degeneration and regeneration, satellite cell proliferation, inflammation, adipocyte infiltration and fibrosis, giving many possibilities for miRNA regulation in diverse cell types. Duchenne muscular dystrophy (DMD) is the most prevalent and most severe of the muscular dystrophies, affecting about 1 in 3 500 live males. This X-linked recessive disease is caused by mutations in the dystrophin gene, which encodes for a large protein that connects the extracellular matrix with the cytoskeleton of muscle. This bridging function is thought to be important to stabilise the sarcolemma.

The mdx mouse – a mouse model for muscle dystrophy

The first investigations of altered miRNA expression in dystrophic skeletal muscle were performed in the most commonly applied mouse model for muscle dystrophy, the mdx mouse. These mice carry a stop codon in the dystrophin gene, mimicking the human situation, but the disease is rather mild in the mice possibly due to a higher regenerative capacity compared with humans. McCarthy et al. analysed with qPCR the expression of three muscle-specific miRNAs, miR-206, miR-1 and mi-133, and found a four- to five-fold increase of miR-206 in diaphragm with no major regulation in soleus or plantaris muscles [200].

Muscle-specific miR-1 and miR-133a showed no major regulation in any of the three muscle groups. The induction of miR-206 was most likely posttranscriptional since the primary transcript of this miRNA, pri-miR-206, was also upregulated. The isolated induction of miR-206 in the diaphragm was not completely surprising since the diaphragm represents the most severely affected muscle group in this mouse model. However, it was later shown that miR-206 is induced, while miR-1 is downregulated, also in other muscle groups of the mdx mice [203].

Regulation of miRNAs in patients with muscle dystrophy

Using miRNA microarrays, Eisenberg et al. identified no fewer than 151 miRNAs that are upregulated and 28 that are downregulated in 10 major muscle disorders [201]. Interestingly, with the exception of five miRNAs, the expression of these miRNAs was specific to individual disease groups. A significant correlation between miRNAs and their targets was observed in DMD patients and patients with Miyoshi myopathy, indicating that miRNAs could be also functionally relevant in muscle diseases. Messenger RNA –miRNA modules regulated in both muscle diseases were extracellular matrix processes and cytoskeletal organisation, indicating that these could be common functional clusters in dystrophic muscles regulated by miRNAs. Using qPCR, Greco et al. compared the levels of 250 adductor muscle miRNAs in mdx versus wild-type mice and identified 36 miRNAs that were regulated significantly [202]. Importantly, 11 miRNAs were identified as a common miRNA signature between mdx mice and DMD patients including miR-1, which was about three-fold downregulated, and miR-206, which was four- to nine-fold upregulated. Regulation of only three miRNAs was overlapping with the report from Eisenberg et al (miR-222, miR-335, miR-29c). Cacciarelli et al. ran low-density qPCR arrays on mdx and wild-type muscles, and detected as the most prominent changes the downregulation of miR-1, miR-133a, miR-29c and miR-30 and the induction of miR-206 [203]. Downregulation of miR-1 and miR-29c was attributed to transcriptional repression by histone deacetylase 2 (HDAC2). Importantly, this study also addressed the intramuscular localisation of miR-1 and miR-206. Localisation of miR-206 was restricted to newly-formed regenerating myofibres characterised by a centralised nucleus, while miR-1 showed intense signals in all mature fibres. These results indicated that increased expression of miR-206 is caused by the presence of newly formed fibres, while the downregulation of miR-1 is the consequence of a decrease in adult myofibres. The same group later discovered that the muscle-specific miRNAs are released into the blood of Duchenne patients and that their levels correlated with disease severity [204]. Cacciarelli et al. also identified a nonmuscle specific miRNA, miR-31, that colocalised with miR-206 in regenerating fibres and was also strongly induced in mdx muscles and DMD patient samples [205].

The miR-206 knockout mouse

The first evidence for a functional role of a regulated miRNA in dystrophic muscles *in vivo* came from Liu et

al., who deleted miR-206 in mice during embryonic development and crossed these mutants with the mdx mice [206]. The loss of miR-206 resulted in a more severe dystrophic phenotype in the mdx mice with creatine kinase activity, a marker of muscle damage, being eight-fold higher in the mutants than in the control mice. These results indicate that the upregulation of miR-206 in dystrophic muscle could serve as a protection mechanism to facilitate the formation of new fibres during muscle regeneration. Interestingly, miR-206 was also found to be significantly upregulated in skeletal muscles of the SOD1^{G93A} mouse model of ALS [150, 152], as well as in skeletal muscles of ALS patients [151]. It has been shown that miR-206 promotes compensatory mechanisms that lead to regeneration of neuromuscular junctions, partially reinnervating muscles and resulting in delayed disease progression in the SOD1^{G93A} mouse model [150], further supporting the idea that upregulation of miR-206 in regenerating muscle fibres is protective and its overexpression in skeletal muscles of ALS patients could delay muscle weakness and possibly slow down disease progression.

Potential for miRNAs as biomarkers in muscle dystrophy

Together these studies provide evidence that miRNAs are regulated in dystrophic muscles in animal models and patients. The overlap of regulated miRNAs between these studies is rather small, probably as a result of the different muscle groups that had been investigated or different techniques with which miRNAs were quantified (oligonucleotide arrays, qPCR, northern blotting). However, downregulation of miR-1 and upregulation of miR-206 have been repeatedly confirmed. These studies also demonstrate that miRNAs in muscle tissue or serum could be interesting markers for disease severity in muscle dystrophy. miRNAs in serum could be envisioned as biomarkers to monitor noninvasively clinical trials or to identify patients, who would benefit from interventions. The example of miR-206 highlights the idea that miRNAs could also serve as new therapeutic targets for muscle diseases.

Role of miRNA in cardiovascular disease

Several miRNAs have been identified as critical modulators of cardiovascular development and pathophysiology [207]. The important role of miRNAs in the cardiovascular system has provided novel perspectives on the pathophysiology of cardiovascular disease and has revealed interesting potential novel therapeutic targets [208, 209].

miRNA and cardiac function

Cardiac deletion of enzymes required in the biogenesis of miRNAs, such as Dicer, resulted in dilated cardiomyopathy and premature lethality, supporting a critical role of miRNA biogenesis for cardiac function and structure [210, 211]. By using a high-throughput functional screening approach, Wahlquist et al. have recently identified miR-25 as a potent regulator of calcium uptake kinetics in cardiomyocytes *in vitro* (out of 875 miRNAs tested) and observed that miR-25 was upregulated in heart failure, in both mice

and humans [212]. Moreover, overexpression of miR-25 *in vivo* resulted in a reduced contractile cardiac function, whereas injection of an antisense oligonucleotide against miR-25 (anti-miR-25) markedly prevented progression of established heart failure in a mouse model and improved cardiac function and survival [212]. Furthermore, miR-34a was reported to be induced in ageing hearts and *in-vivo* silencing of miR-34a reduced age-associated cardiomyocyte cell death, cardiac fibrosis and promoted recovery of cardiac function after myocardial infarction [213]. Interestingly, miR-34a is upregulated in blood mononuclear cells of patients with sporadic AD [54], which may also represent a consequence of aging.

miRNA in cardiac development and stem cells

Dynamic regulation of miRNA is involved in developmental biology, for example, differentiation of embryonic stem cells towards a cardiomyocyte fate, and *in-vitro* studies have shown that miR-1, together with miR-499, are upregulated in the differentiation of human embryonic stem cells and cardiac progenitor cells toward cardiomyocytes. [214, 215] Overexpression of these miRNAs enhances differentiation toward a cardiomyocyte fate [214, 215].

miRNA and atherosclerotic vascular disease

Several recent reports have revealed that the human sterol-regulatory-element-binding-protein genes SREBF1 and SREBF2 harbour two intronic miRNAs, miR-33b and miR-33a, respectively, that modulate homeostasis of cholesterol and triglycerides, which are critical for the atherosclerotic disease process [209, 216]. Anti-miR33-treated mice showed reduced atherosclerotic plaque size and lipid content and decreased inflammatory gene expression [217].

Pharmacological inhibition of miR-33 using a subcutaneously injected, locked nucleic acid (LNA)-modified anti-miR in a nonhuman primate metabolic disease model lowered very low-density lipoprotein triglycerides [218] and resulted in limited atherosclerosis progression in a mouse model of atherosclerosis [217].

We and others have observed a critical role of miR-126, which is particularly expressed in endothelial cells, for ischaemia-induced neovascularisation, for example, after myocardial infarction and in atherosclerotic vascular disease [219, 220]. In patients with chronic heart failure, miR-126 levels were markedly reduced in circulating endothelial repair-promoting cells. Moreover, administration of miR-126 promoted endothelial cell proliferation at predilection sites and reduced experimental atherosclerosis, suggesting that miR-126 may represent a potential novel therapeutic target to prevent progression of atherosclerotic vascular disease, the main cause of death in developed countries [220]. Notably, miR-126 limits leucocyte adherence to endothelial cells, at least in part by suppressing endothelial vascular cell adhesion molecule 1 (VCAM-1) expression, suggesting that miR-126 may limit vascular inflammation [221].

Role of miRNA in the pathogenesis of inflammation

MiRNA in autoimmune and inflammatory diseases

The immune system protects the organism from attack by microorganisms such as bacteria, viruses and parasites. Whereas the local inflammation at the site of infection and elimination of infected cells is necessary for defense of the individual, failure to control inflammation once microorganisms are cleared can have devastating consequences. It is, therefore, important to understand the molecular mechanisms regulating immune reactions and their defects in the various autoimmune diseases. The recognition of noncoding RNAs, in particular miRNAs, as a new principle of regulation of gene-expression, has been a major advance. Dysregulation of miRNA has been linked to cancer, cardiovascular, infectious and autoimmune diseases. Important pathways in the innate as well as the adaptive immune system are under control of miRNAs. As one miRNA has a multitude of targets and acts in a tissue specific way, the complexity of this regulatory system is considerable. Increasing evidence indicates that the expression patterns of miRNAs are dysregulated in a variety of autoimmune diseases. Whereas many different miRNAs have been reported to be either up- or downregulated in specific autoimmune diseases, an impact of these expression changes on the development of autoimmune diseases has been demonstrated only in a few instances so far.

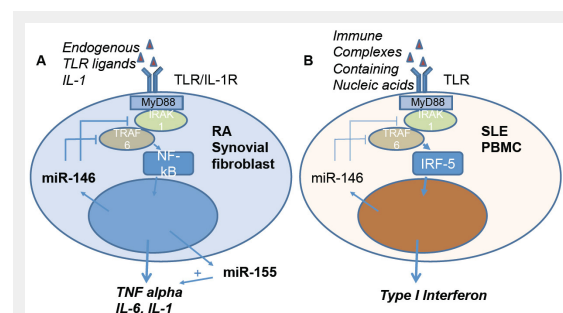


Figure 3

Regulatory role of miR-146 and miR-155 on inflammation in activated synovial fibroblasts of patients with rheumatoid arthritis (A) and on type I interferon production in PBMC of patients with SLE (B). (A) Activation of synovial fibroblasts by endogenous Toll-like receptor (TLR) ligands or interleukin 1 (IL-1) initiates a signalling cascade via nuclear factor kappa B (NF-κB) resulting in expression of proinflammatory cytokines, such as tumour necrosis factor alpha (TNF-α). Overexpression of TNF-α is a hallmark of rheumatoid arthritis. In activated synovial fibroblasts miR-155 expression is induced, further enhancing TNF-α production. In contrast, miR-146 has a modulatory role by inhibiting the signalling molecules interleukin 1 associated receptor kinase 1 (IRAK1) and TNF-receptor accessory factor 6 (TRAF 6). (B) Regulatory role of miR-146 in SLE PBMC. In contrast to the overexpression of miR-146 in RA, in SLE miR-146 levels are decreased, resulting in an increased activation of IRF-5 and expression of type 1 interferon.

IRF-5 = interferon regulatory factor 5; PBMC = peripheral blood mononuclear cells; RA = rheumatoid arthritis; SLE = systemic lupus erythematosus

Rheumatoid arthritis

First reports on miRNA expression in rheumatoid arthritis appeared in 2008, describing upregulation of miR-155 and -146 in the rheumatoid synovium [222, 223]. Expression of miR-155 was shown to be induced by lipopolysaccharide and polyinosinic:polycytidylic acid (poly-I:C) in a MyD88-dependent fashion [224]. Macrophages overexpressing miR-155-produced significantly more tumour necrosis factor- α (TNF- α) and miR-155 transgenic mice showed increased sensitivity to lipopolysaccharide-induced septic shock, whereas miR-155 deficient mice failed to produce TNF- α [45, 46]. Also, miR-155 deficient mice were shown to be resistant to collagen-induced arthritis, whereby the generation of autoreactive B and T cells was prevented. The example of miR-155 demonstrates that a single miRNA can have a profound impact on the development of an autoimmune disease (fig. 3). Contrary to miR-155, miR-146 was shown to have an anti-inflammatory role. By targeting IRAK-1 and TRAF-6 miR-146 is a negative regulator of NF- κ B activation. Accordingly, miR-146 deficient mice develop a fatal immune-mediated disease and tumours in lymphoid organs [47]. Administration of miR-146 in mice with established collagen-induced arthritis reduced bone destruction, but had no significant effect on inflammation [44]. Interestingly, a polymorphism in the 3'UTR of the IRAK-1 gene, a target of miR-146, is associated with the susceptibility to rheumatoid arthritis [225]. A number of additional miRNAs have been shown to be dysregulated in rheumatoid arthritis synovial cells. Most of these studies are however mainly descriptive and provide limited information on the functional impact of the miRNA as well as the regulation of their expression.

Systemic lupus erythematosus

Studies of miRNA in systemic lupus erythematosus (SLE) have focused primarily on miR-146a. In peripheral blood mononuclear cells of patients with SLE, miR-146 was found to be downregulated, and expression of miR-146 was inversely correlated with the disease activity. Overexpression of miR-146 reduced type I interferon production by targeting IRF-5 and STAT-1 [226]. Therefore it was proposed that deficient miR-146 expression may be one of the causal factors of type I interferon pathway activation, characteristically seen in SLE. In support of this hypothesis, a functional promoter variant of miR-146a was found to confer risk for developing SLE [227]. Treatment of lupus-prone BXSB mice with virus-like particles containing miR-146 significantly reduced autoantibody production and could ameliorate SLE progression [228]. Analyses of expression profiles of miRNA in kidney tissue of patients with SLE revealed that miRNA expression is organ and cell-type specific, as expression differed from peripheral blood mononuclear cells of SLE patients. In contrast to the findings in peripheral blood mononuclear cells, miR-146a was found increased in glomerular tissue. In rodent models of SLE, various additional miRNAs have been found to contribute to disease. Both miR-21 and miR-214 were shown to be upregulated in renal tissue and upon stimulation of rat epithelial cells with transforming growth factor- β (TGF- β) *in vitro*. Inhibition of TGF- β signalling resulted in a downregulation of miR-21 and -214 and in-

hibition of epithelial-mesenchymal transition and collagen type I production. This suggests that TGF- β -induced miR-21 and miR-214 expression may contribute to extracellular matrix production and mesangial proliferative glomerulonephritis [229].

miR-21 was also found to be upregulated in systemic sclerosis skin fibroblasts and correlated with disease activity. A profibrotic effect of TGF- β -induced miR21 was postulated by negatively regulating its target Smad7 [230].

Interestingly, it has been shown that patients with connective tissue diseases may produce antibodies against proteins of the miRNA pathway, which localise in the cytoplasm in the so-called GW-bodies. Anti-GWB-antibodies have been identified in patients with SLE, Sjogren's syndrome and rheumatoid arthritis and seem to be associated with neurological symptoms [231]. The functional relevance of these autoantibodies is still unclear.

Inflammatory bowel disease

In 2008 alterations in miRNA expression in inflammatory bowel disease were described for the first time [232]. Analyses of miRNA expression profiles of intestinal biopsy tissue as well as of peripheral blood mononuclear cells have revealed distinct profiles in ulcerative colitis and Crohn's disease. However, information on the functional impact of these miRNAs is still lacking to a large extent. Decreased levels of miR-7 in inflamed colonic tissue in Crohn's disease have been reported to correlate with upregulated expression of CD98, its target, which controls proliferation and differentiation of enterocytes [233]. A recent study has shown overexpression of miR-196 in intestinal epithelial cells in inflamed Crohn's disease. Interestingly, miR-196 was found to bind to the protective variant of the immunity-related GTPase M (IRGM) but not a mutated IRGM which was shown to be highly associated with Crohn's disease in European populations [234]. Similarly, it has been demonstrated that a variant of the IL-23R gene, highly associated with inflammatory bowel disease, results in the loss of a binding site for the miRNAs let-7e and let-7f, with increased expression of IL-23R *in vitro*. These results demonstrate possible links of miRNA dysregulation and pathogenesis of inflammatory bowel disease [235].

In summary, in autoimmune and autoinflammatory diseases a dysregulation of miRNA expression has been demonstrated which can have a profound impact on the pathogenesis of these diseases. Therefore, miRNAs are attractive targets for immunomodulatory therapeutic intervention. Moreover, miRNAs hold great promise as markers of disease activity and prognosis and to guide therapeutic intervention (for review [236]).

Outlook on translation of miRNA research into the clinics

Clearly, basic research provided us with breakthrough discoveries of miRNA biogenesis and the crucial function of miRNAs as genome regulators. Animal research using various mouse disease models was an important step that revealed that miRNAs indeed do change in disease. Later, it was recognised that changes in miRNA levels could be either a consequence or a direct trigger of the disease and it

is now well established that changes of levels in numerous miRNAs reflect the disease onset and progress, and that could even be used for presymptomatic diagnosis and prognosis. Moreover, targeting the dysregulated miRNAs represents a new and very potent therapeutic intervention.

It is, however, important to point out that the results obtained from animal models are very often not directly replicated in the clinic. This may be owing to the inherent variability in human samples and it is obvious that all screening conditions (including sample source, size and preparation, screening techniques, data collection, analysis, validation and interpretation) need to be standardised across all patients and study sites in order for the miRNAs to become reliable disease biomarkers.

Unbiased screening using next generation RNA sequencing enables us to screen all known miRNAs, thus eliminating the possibility that the most affected miRNAs would be missed. Finally, given the large number of miRNAs, each regulating hundreds of mRNAs, it is possible that multiple miRNAs will become markers of certain disease processes rather than a single miRNA. Once such data are available, algorithms that would compare them across different patient groups and/or diseases may reveal common molecular mechanisms that would enable us to identify potential asymptomatic, yet already ongoing, pathological processes that may currently be missed during routine diagnostic procedures. This could lead to early therapeutic intervention that may involve direct targeting of the known, pathologically dysregulated miRNA(s), as well as other targets. We foresee that in the future, all this will be feasible from a single drop of body fluid, such as blood or CSF.

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Figures (large format)

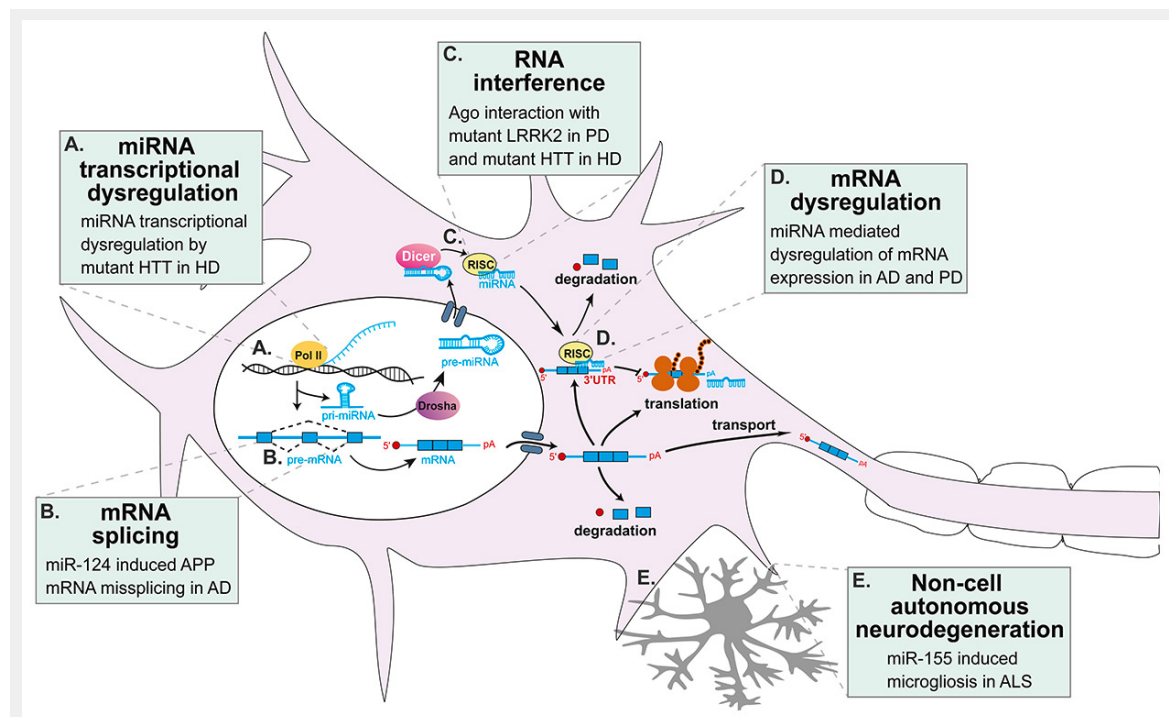


Figure 1: miRNA alterations in neurodegenerative diseases. Numerous alterations in miRNA biogenesis and function were found to contribute to the pathogenesis of several neurodegenerative diseases including: (A) In HD, miRNA transcription is disturbed as mutant huntingtin does not bind REST (which is normally bound to wild-type huntingtin), allowing the formation of NRSE thus repressing the transcription of neuronal miRNAs [135, 181, 183]. Mutant huntingtin also binds to p53 dysregulating p53-mediated transcription of miRNAs [16, 188]. (B) Downregulation of miR-124 has been found to cause missplicing of APP in AD [42]. (C) Ago has been found to be sequestered by mutant huntingtin and accumulated into stress granules in HD suggesting impaired miRNA-mediated mRNA regulation [189–191]. Interestingly, mutant LRRK2 also interacts with Ago, impairing mRNA regulation by miRNAs [72]. (D) Expression of APP and BACE1 in AD [37–41, 48–52] and SNCS and LRRK2 in PD [71–73] has been described to be specifically affected by their regulating miRNA(s) that are disturbed in these diseases. (E) Non-cell autonomous neurodegeneration is now well recognized in ALS and miR-155 seems to play a major role in the pathologic microgliosis [43, 163, 165].

3'UTR = 3' untranslated region; AD = Alzheimer's disease; ALS = amyotrophic lateral sclerosis; APP = amyloid precursor protein; BACE-1 = β -secretase 1; HD = Huntington's disease; HTT = huntingtin gene; LRRK2 = leucine-rich repeat kinase 2; miRNA = microRNA; mRNA = messenger RNA; NRSE = neuron restrictive silencer element; PD = Parkinson's disease; pri-miRNA = primary miRNA; REST = RE1-silencing transcription factor; RISC = RNA-induced silencing complex

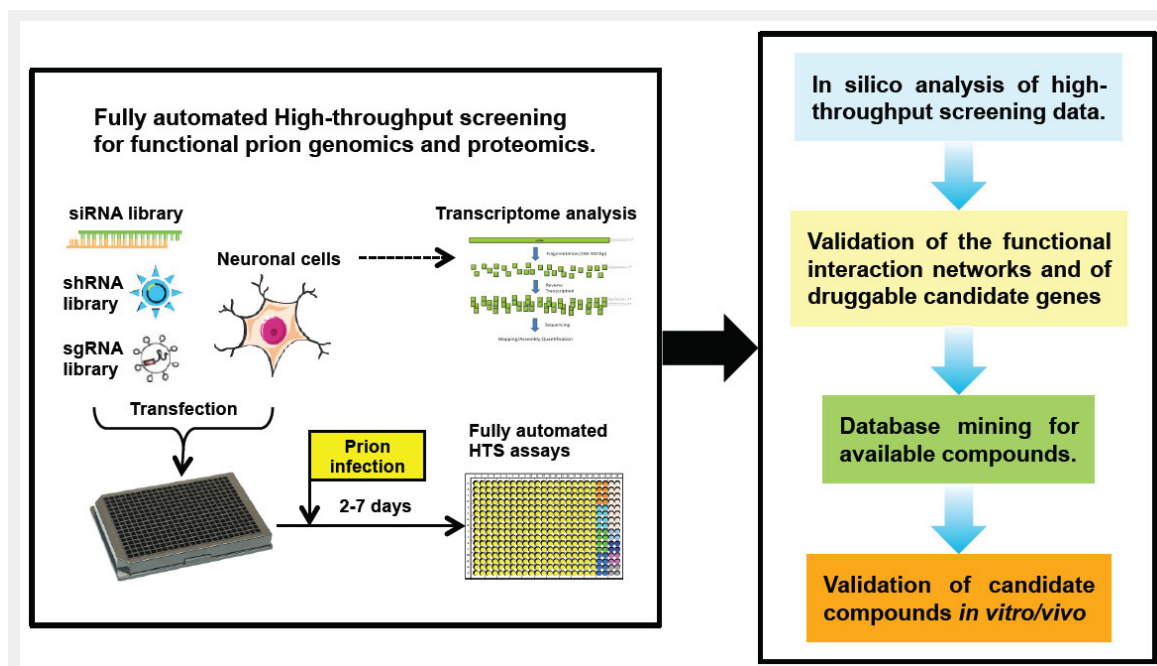


Figure 2: Proposed new strategies for high throughput small RNA screens to discover cell signalling mechanisms and novel therapeutic target for prion disease.

HTS = high throughput screening; sgRNA = single guide RNA; shRNA = short hairpin RNA; siRNA = small interfering RNA

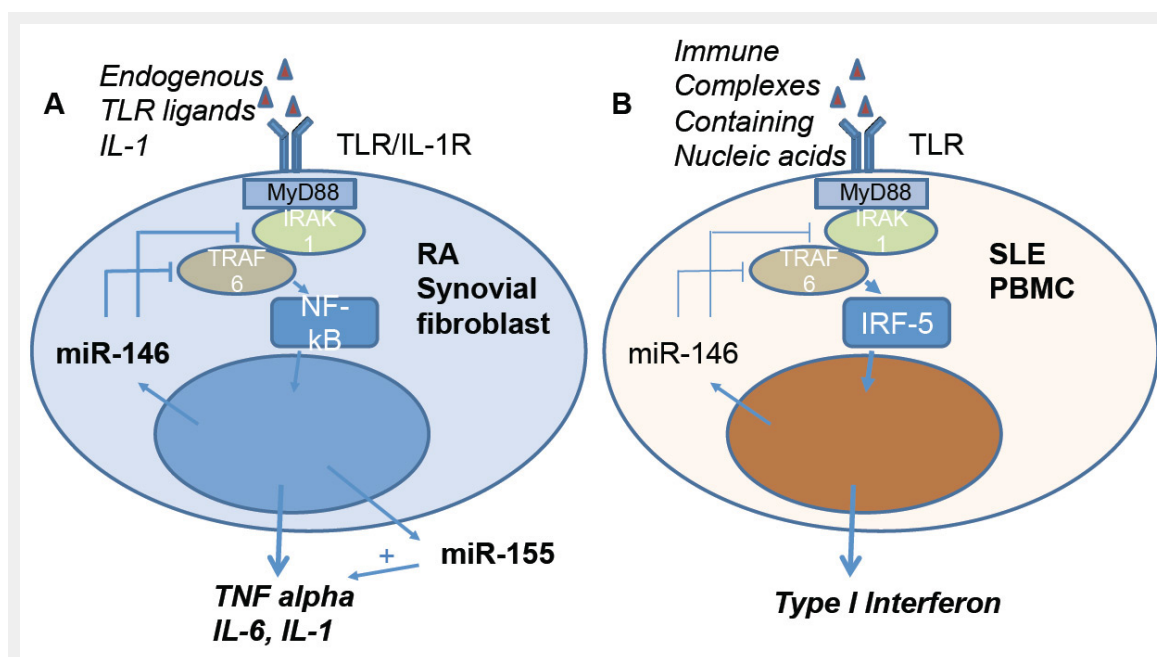


Figure 3: Regulatory role of miR-146 and miR-155 on inflammation in activated synovial fibroblasts of patients with rheumatoid arthritis (A) and on type I interferon production in PBMC of patients with SLE (B). (A) Activation of synovial fibroblasts by endogenous Toll-like receptor (TLR) ligands or interleukin 1 (IL-1) initiates a signalling cascade via nuclear factor kappa B (NF-κB) resulting in expression of proinflammatory cytokines, such as tumour necrosis factor alpha (TNF-α). Overexpression of TNF-α is a hallmark of rheumatoid arthritis. In activated synovial fibroblasts miR-155 expression is induced, further enhancing TNF-α production. In contrast, miR-146 has a modulatory role by inhibiting the signalling molecules interleukin 1 associated receptor kinase 1 (IRAK1) and TNF-receptor accessory factor 6 (TRAF 6). (B) Regulatory role of miR-146 in SLE PBMC. In contrast to the overexpression of miR-146 in RA, in SLE miR-146 levels are decreased, resulting in an increased activation of IRF-5 and expression of type 1 interferon.

IRF-5 = interferon regulatory factor 5; PBMC = peripheral blood mononuclear cells; RA = rheumatoid arthritis; SLE = systemic lupus erythematosus